

A Proteomic Study of the Response to Salinity and Drought Stress in an Introgression Strain of Bread Wheat^{*}

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The effect of drought and salinity stress on the seedlings of the somatic hybrid wheat cv. Shanrong No. 3 (SR3) and its parent bread wheat cv. Jinan 177 (JN177) was investigated using two-dimensional gel electrophoresis and mass spectrometry. Of a set of 93 (root) and 65 (leaf) differentially expressed proteins (DEPs), 34 (root) and six (leaf) DEPs were cultivar-specific. The remaining DEPs were salinity/drought stress-responsive but not cultivar-specific. Many of the DEPs were expressed under both drought and salinity stresses. The amounts of stress-responsive DEPs between SR3 and JN177 were almost equivalent, whereas only some of these DEPs were shared by the two cultivars. Overall, the number of salinity-responsive DEPs was greater than the number of drought-responsive DEPs. And most of the drought-responsive DEPs also responded to salinity. There are both similarities and differences in the responses of wheat to salinity and drought. A parallel transcriptomics analysis showed that the correlation between transcriptional and translational patterns of DEPs was poor. The enhanced drought/salinity tolerance of SR3 appears to be governed by a superior capacity for osmotic and ionic homeostasis, a more efficient removal of toxic by-products, and ultimately a better potential for growth recovery. *Molecular & Cellular Proteomics* 8:2676–2686, 2009.

Soil salinity and drought are the two most common abiotic stresses constraining crop growth and productivity (1). As a result, the development of improved levels of tolerance to these stresses has become an urgent priority for many crop breeding programs. In parallel, much research effort is being applied to gain a better understanding of the adaptive mechanisms used by plants to combat abiotic stress. High throughput genetic screening platforms have delivered substantial insights into these responses and have defined a number of the cellular and molecular processes involved in the response to abiotic stress (2, 3). The emerging picture is that of a complex gene network, centered largely on signal transduction.

The current focus is now shifting from genomics to proteomics analysis because many gene products are subject to post-translation modification, which cannot be detected by transcriptomics analyses. A number of recent studies have attempted to describe changes to the proteome in response to salinity and/or drought stress (1, 4–6). The primary effect of drought is to generate osmotic stress, whereas salinity induces osmotic stress more indirectly by its effect on the ionic homeostasis within the plant cell (7). Thus, it is unsurprising that there is an element of both commonality and distinctness in the response mechanisms to salinity and drought stresses. When *Arabidopsis thaliana* cell suspension cultures were exposed to either osmotic or salinity stress, it was possible to define a large number of responsive proteins (6). Similarly, a proteomics analysis of rice roots and leaves exposed to either salinity or drought stress led to the identification of several stress-responsive proteins (8). However, the global response to salinity or drought stress remains largely unexplored.

Wheat is one of the world's major crops and has been subjected to intensive breeding and selection for about a century. The bulk of the selection effort to date has been directed to improving grain yield, end use quality, and disease resistance. With increasing pressure on water supply, a major shift is now underway to improve its level of abiotic tolerance. Recently, we have released the bread wheat cultivar Shanrong No. 3 (SR3)¹ with traits of salinity and drought tolerance. SR3 is a wheat introgression line containing alien chromatin from tall wheatgrass via asymmetric somatic hybridization between parent bread wheat JN177 and its wild relative tall wheatgrass (*Thinopyrum ponticum* Podp) (9–11), one of the most salinity-tolerant of all monocotyledonous species (12). The seedling root proteomes of SR3 and JN177 have been compared under both non-stressed and salinity-stressed conditions (11). This comparison led to the identification of 114 differentially expressed proteins (DEPs), and the presumed function of many of these could be defined on the basis of homology with orthologous gene products. However, the fragmentary results did not bring about an overall profile of

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¹ The abbreviations used are: SR3, Shanrong No. 3; DEP, differentially expressed protein; PMF, peptide mass fingerprinting; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; ROS, reactive oxygen species; JN177, Jinan 177; 2-DE, two-dimensional gel electrophoresis; EST, expressed sequence tag.

the systematic causes of the higher salt and drought tolerance of SR3 than its parent JN177. The present study was intended to extend these results to compare the leaf and root proteomes of SR3 and JN177 under both drought and salinity stresses.

MATERIALS AND METHODS

Salinity and Drought Treatments—Wheat seedlings were grown hydroponically following the methods described elsewhere (11). The salinity and drought treatments were applied to seedlings of SR3 and JN177 at the two-leaf stage by adding either 200 mM NaCl or 18% (w/v) polyethylene glycol 6000 to the half-strength Hoagland's culture solution. Control plants remained in culture solution without any stress-inducing additive. After 24 h of exposure, the roots and leaves were harvested. All analyses were performed on three replicated plant samples.

Biomass Measurement and Biochemical Characterization—The measurement of seedling biomass and Na^+/K^+ ratio was performed as described previously (11). The net photosynthesis rate and transpiration rate of the second seedling leaf were assessed using an LI-6400XT portable photosynthesis system (LI-COR Biosciences) under $800 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light and at a temperature of 27°C and a relative humidity of 40%. The content of soluble sugars was quantified by the sulfuric acid-anthrone method (13), and the content of sucrose was quantified by the resorcinol method (14). Leaf chlorophyll was extracted by acetone, and the contents of the *a* and *b* types were determined spectrophotometrically at 663 and 645 nm, respectively.

Protein Extraction, Two-dimensional Gel Electrophoresis (2-DE), In-gel Digestion, and MS Analysis—Protein extraction was carried out following methods described elsewhere (11) with minor modifications. Briefly, the powder of root and leaf samples was suspended in 10% (w/v) trichloroacetic acid, acetone containing 1 mM PMSF and 0.07% (w/v) β -mercaptoethanol and held at -20°C for 1 h. After centrifugation and rinsing, the vacuum-dried pellets were dissolved in 500 μl of lysis solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 1 mM PMSF, and 0.5% (v/v) Bio-Lyte (Bio-Rad). Insoluble materials were removed by centrifugation, and the protein concentration of the sample was quantified using the Bradford method (15). About 130 μg of protein was separated by loading the sample on a 17-cm pH 5–8 linear pH gradient IPG strip (Bio-Rad) and subjecting to electrophoresis for 100 kV-h. The second electrophoretic dimension was by 10% SDS-PAGE. The signal was visualized by silver staining. The analysis was based on total densities of gels with the parameter of percent volume, and a significant difference in expression of a spot was declared if the mean abundance varied more than 2-fold using a *t* test.

DEPs detected in silver-stained gels were excised from replica gels stained by Coomassie Brilliant Blue. After destaining, reduction, and alkylation, in-gel digestion was performed by incubation at 37°C for 16 h in trypsin solution according to the Bio-Rad manual. Digested peptide fragments were thoroughly recovered; vacuum-dried; resuspended in 3 μl of 0.5% (w/v) TFA, 30% (v/v) ACN; and subjected to MALDI-TOF-TOF mass spectroscopy on a 4700 Plus MALDI TOF/TOFTM analyzer (Applied Biosystems). Peak list generation and peak picking for PMF and MS/MS data were conducted with GPS Explorer (Applied Biosystems 2006). Parameters and thresholds used for peak picking were as follows: signal/noise threshold >20 , resolution $>10,000$, and means of calibrating each spectrum as external calibration. PMFs and MS/MS data were used to derive protein identity using the MASCOT search engine (Matrix Science) applied to the NCBI nr, MSDB, and Swiss-Prot databases. The PMF acceptance criterion is probability/E-value-based scoring. The search parameters

were as follows: one trypsin miscleavage permitted, MH^+ mass values, and monoisotopic. Other search parameters concerning the release versions of databases, modifications such as carbamidomethyl of Cys and oxidation of Met, and mass tolerance for precursor and fragment ions are stated in the protein identification tables (supplemental Tables 2S1 and 2S4).

cDNA Microarray Assay and RT-PCR—Total root RNA and leaf RNA were isolated from both control (non-stressed) and stressed seedlings for cDNA microarray and RT-PCR assays using the TRIzol[®] reagent (Invitrogen) following the manufacturer's protocol. The cDNA microarray assay was performed as described elsewhere (11). The RT-PCR conditions consisted of an initial incubation of 95°C for 60 s followed by 28 cycles of 95°C for 30 s, annealing at 50 – 60°C for 30 s, and 72°C for 40 s and ending with a 7-min incubation at 72°C . To design RT-PCR primers, the sequence of each DEP was first used as a tBLASTn search term against wheat ESTs. The best aligned EST, as well as its corresponding UniGene, was selected; all ESTs clustering with this UniGene were electronically assembled; and the assembled sequence was used for primer design. The primer sequences for the RT-PCR assays are given in supplemental Table S1.

RESULTS

Plant Growth and Physiological Response to Stress—Drought and salinity stresses both reduced SR3 and JN177 seedling fresh weight (Table I). However, the adverse effect on JN177 was greater than on SR3 because SR3 seedling fresh weight was 16% (drought) and 11% (salinity) higher than that of JN177 seedlings. Dry weight behaved in the same way. SR3 produced longer roots in the control and both the stress treatments. Salinity stress significantly reduced chlorophyll *a* and *b* contents in both cultivars, but the JN177 chlorophyll content was significantly lower than that of SR3 under salinity stress. The net photosynthesis rate of JN177 was more inhibited by stress than was that of SR3. Under drought stress, the transpiration rate of SR3 and JN177 fell by 34 and 16%, respectively; whereas under salinity stress, the decrease was even greater (52 and 36%, respectively). The leaf soluble sugar content in SR3 rose by 11% (drought) and 17% (salinity) but remained unchanged in JN177 so that, under salinity stress, the leaf sucrose content of SR3 was 51% more than that of JN177. In the root, both stresses reduced the soluble sugar content of JN177 (by 30–46%), whereas in SR3, salinity stress reduced the soluble sugar content by 28%, but drought stress had no statistically significant effect on this parameter. Under salinity stress, the Na^+ content of the leaves/stems of JN177 and SR3 seedlings both increased nearly 6-fold and in the roots by nearly 6- and 8-fold, respectively. Salinity stress reduced the K^+ content of JN177 leaves/stems and roots by 36 and 87%, respectively, and those of SR3 by 17 and 85%, respectively. Drought stress altered the Na^+ and K^+ contents in SR3 and JN177 to a lesser extent. The K^+/Na^+ ratio in the leaves/stems and roots were markedly decreased in both SR3 and JN177. The higher ratio in the SR3 leaves is diagnostic of the higher salinity tolerance of this cultivar.

2-DE Maps and Identification of DEPs—The root 2-DE map (Fig. 1A) consisted of at least 1031 reproducible protein spots of which 93 were classified as DEPs on the basis of six

TABLE I
Physical and chemical measurements of JN177 and SR3 plants grown under non-stressed and stressed (drought or salinity) conditions

Indices	Jinan 177			Shanrong No. 3		
	Control	Drought	Salinity	Control	Drought	Salinity
Fresh weight (g)	0.563 (a) ^a	0.324 (b)	0.361 (b)	0.552 (a)	0.376 (b) ^b	0.418 (b) ^b
Dry weight (g)	0.047 (a)	0.037 (b)	0.038 (b)	0.051 (a)	0.043 (b) ^b	0.045 (b) ^b
Root length (cm)	21.3 (a)	15.7 (b)	13.6 (b)	23.7 (a) ^b	19.7 (b) ^b	17.0 (b) ^b
Chlorophyll a (mg·g ⁻¹ DW ^c)	13.91 (a)	13.04 (a)	7.48 (b)	14.48 (a)	13.76 (a)	9.48 (b) ^b
Chlorophyll b (mg·g ⁻¹ DW)	5.33 (a)	4.93 (a)	3.45 (b)	5.51 (a)	5.09 (a)	3.22 (b)
Photosynthetic rate (mm·m ⁻² ·s ⁻¹)	11.71 (a)	3.75 (b)	4.05 (b)	13.15 (a)	3.97 (b)	6.11 (c) ^b
Transpiration rate (mm·m ⁻² ·s ⁻¹)	2.34 (a)	1.96 (b)	1.49 (b)	2.31 (a)	1.52 (b) ^b	1.12 (c)
Soluble sugar in leaf (mg·g ⁻¹ DW)	56.40 (a)	60.36 (a)	55.03 (a)	59.11 (a)	65.73 (b) ^b	69.42 (b) ^b
Soluble sugar in root (mg·g ⁻¹ DW)	49.18 (a)	39.52 (b)	41.55 (b)	45.77 (a)	41.17 (b)	42.78 (ab)
Sucrose in leaf (μg·g ⁻¹ DW)	28.95 (a)	38.73 (b)	35.44 (b)	28.33 (a)	41.78 (b)	53.40 (c) ^b
Sucrose in root (μg·g ⁻¹ DW)	32.98 (a)	22.79 (b)	20.99 (b)	34.03 (a)	31.43 (a) ^b	24.43 (b)
Na ⁺ in leaf/stem (mm·g ⁻¹ DW)	0.284 (a)	0.484 (b)	1.967 (c)	0.272 (a)	0.468 (b)	1.798 (c)
Na ⁺ in root (mm·g ⁻¹ DW)	0.165 (a)	0.203 (a)	1.131 (b)	0.142 (a)	0.194 (a)	1.229 (b) ^b
K ⁺ in leaf/stem (mm·g ⁻¹ DW)	2.088 (a)	1.807 (a)	1.34 (b)	1.903 (a)	1.761 (ab)	1.580 (b)
K ⁺ in root (mm·g ⁻¹ DW)	1.074 (a)	0.935 (a)	0.141 (b)	1.153 (a)	1.066 (a)	0.174 (b)
K ⁺ /Na ⁺ in leaf/stem	7.673 (a)	3.851 (b)	0.686 (c)	7.068 (a)	3.806 (b)	0.880 (c) ^b
K ⁺ /Na ⁺ in root	6.518 (a)	4.707 (a)	0.124 (b)	8.428 (a)	5.599 (a)	0.142 (b)

^a Letters (a, b, and/or c) indicate statistical differences among non-stressed, drought-stressed, and salinity-stressed JN177 or SR3 using one-way analysis of variance least significant difference analysis.
^b Indicates significant differences between JN177 and SR3 under non-stressed, drought-stressed, or salinity-stressed conditions using *t* test (*p* < 0.05).
^c DW, dry weight.

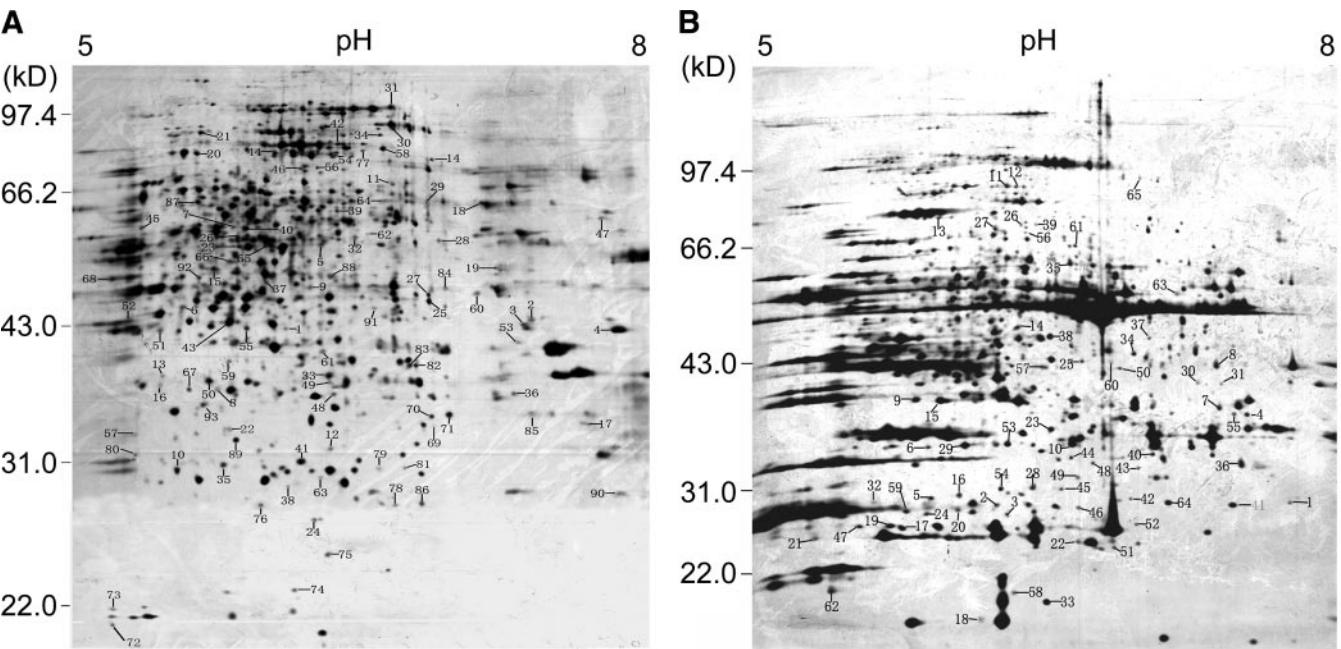


FIG. 1. 2-DE reference maps of wheat seedling root and leaf proteomes. A, root map; B, leaf map. The numbered leaf and root protein spots were labeled as L and R, respectively, in the main text and tables.

replicated separations. MALDI-TOF MS analysis was able to identify 76 of these (supplemental Table 2S1), and MALDI-TOF/TOF MS confirmed and corrected the identity of 41 as well as identified the other 10 DEPs (which were not recognized by MALDI-TOF MS) (Table II and supplemental Tables 2S2 and 2S3). The leaf proteome (Fig. 1B) consisted of at least 950 proteins of which 65 were DEPs. The identity of 55 DEPs was obtained by MALDI-TOF MS (supplemental Table 2S4),

and 24 of these and another three (which were not recognized by MALDI-TOF MS) were confirmed and identified by MALDI-TOF/TOF MS (Table II and supplemental Tables 2S5 and 2S6).
Cultivar Proteome Differences—Both the non-stressed and stressed SR3 and JN177 proteomes differed from one another. Root and leaf DEPs were classified into those that were cultivar-specific under either non-stressed or stressed conditions and those that were treatment-specific. In the absence

of stress, there were 49 SR3-specific root (25 up- and 24 down-regulated) and 30 leaf (22 up- and eight down-regulated) DEPs (Table II and supplemental Tables 2S3 and 2S6). These 79 proteins were involved in a wide range of biological processes, including signal transduction, transcription regulation, cell defense, carbon and nitrogen metabolism, and photosynthesis. In the presence of salinity stress, 34 of the 93 root DEPs were cultivar-specific (21 specific to JN177 and 13 specific to SR3) (Table II, supplemental Table 2S3, and Fig. 2). Of the JN177-specific DEPs, 12 were unaffected by stress treatment, seven were up-regulated, and two were down-regulated (Table III). For SR3, the 13 DEPs consisted of five unaffected by stress treatment and six up-regulated and two down-regulated (spots R43 and R54) under stress (Table III and supplemental Table 2S3). Nine (six in SR3 and three in JN177) of the 34 cultivar-specific DEPs were antioxidants. Drought and salinity stresses enhanced the expression of glutathione transferase F4 (spot R41) by 31 and 26-fold, respectively (Table II and Fig. 2). Although peroxidase 10 (spot R59) was induced by stress treatment, the expression level of the peroxidase precursor (spot R71) was unaffected (Fig. 2). The expression of vacuolar proton ATPase subunit E (spot R70) (Fig. 2) and H^+ -transporting two-sector ATPase (spot R23) (both are proton pump-associated proteins) was detectable in SR3 but not in JN177 roots, whereas that of the G protein β -subunit-like protein (spot R33) was found only in the roots of JN177 (Table II and supplemental Table 2S3). The leaf proteomes included fewer cultivar-specific DEPs (supplemental Table 2S6). The expression of catalase (spot L57) was present in SR3 but not in JN177 (Table II).

The treatment-specific DEPs included 59 root and 59 leaf proteins (Table IV and supplemental Table 4S), which could be classified into three types: those that were up-regulated by stress, those that were down-regulated, and those whose expression was unaffected by salinity or drought stress. Most of the root DEPs fell into the first category. Thus, drought stress increased the expression of 32 and 33 DEPs in SR3 and JN177, respectively, and salinity stress led to the up-regulation of 39 and 43 DEPs, respectively. Of them, 21 were up-regulated in both cultivars under drought stress, and 32 were up-regulated in both cultivars under salinity stress. Several signal transduction-associated DEPs showed clearly different patterns of expression. These included the heterotrimeric G protein subunit (spot R67), which was up-regulated in both SR3 and JN177 when exposed to salinity stress; an ethylene receptor (spot R58), which was up-regulated in JN177 but not in SR3; and DWARF3 (spot R72), the expression of which was more than 3-fold stronger in SR3 than in JN177 under salinity stress (Table II and supplemental Table 2S3). Among the leaf stress-responsive DEPs, 33 were up-regulated in drought-stressed SR3, and 34 were up-regulated in JN177 (Table III). Of them, 22 were up-regulated in both cultivars. Under salinity stress, 38 proteins were up-regulated in JN177, and 29 were up-regulated in SR3. The leaf DEPs included

chlorophyll *a/b*-binding apoprotein CP24 precursor (spot L1) and catalase (spot L23), which were both substantially up-regulated in SR3 exposed to salinity stress; whereas a greater presence of the large subunit of Rubisco was detected in JN177 than in SR3 (Table II and supplemental Table 2S6).

Differences between Salinity and Drought Stress Responses—Overall, the number of salinity-responsive DEPs was greater than the number of drought-responsive DEPs. Among the 59 root stress-responsive DEPs, 52 were associated with the salinity response of JN177, and 47 were associated with that of SR3; the number of drought-associated DEPs was only 38 for both cultivars. Leaf DEPs followed the same trend (in JN177, there were 52 salinity-associated DEPs but only 39 drought-associated DEPs; but in SR3, the difference (41 versus 38) was less marked). Most of the root DEPs up-regulated in response to drought stress were also involved in the response to salinity stress; thus, of the 33 JN177 root DEPs, 32 were up-regulated in response to both salinity and drought, whereas in SR3, this applied to 31 of the 32 drought DEPs. Genes that were down-regulated by drought were less likely to be also down-regulated by salinity stress; thus, only one of five (JN177) and three of six (SR3) drought down-regulated DEPs were also down-regulated in response to salinity stress. Among the leaf DEPs, 33 of 34 up-regulated by drought stress in JN177 were also involved in the response to salinity stress. There were five DEPs down-regulated by drought stress in both JN177 and SR3, and four of these, in both cultivars, were also suppressed by salinity stress. There were slightly more drought up-regulated than salinity up-regulated DEPs in SR3 (33 versus 29) (Table II and supplemental Table 2S6).

Among the JN177 stress-responsive DEPs, 16 root and 15 leaf proteins were involved in either the salinity or the drought response but not in both. The SR3 DEPs behaved in the same way except for one root protein (spot R90) (supplemental Tables 2S3 and 4S) and one leaf protein (spot L35) (supplemental Tables 2S6 and 4S), both of which responded differentially to the two stresses. For example, importin α 1b (spot R53) was only up-regulated in salinity-stressed SR3 and JN177 roots, whereas ribosomal protein S8 (spot R49) was only suppressed in the root of both cultivars by drought stress (Table II and supplemental Table 2S3). However, 11 of the 21 JN177-specific root DEPs responded similarly to both stresses (seven were up-regulated, and four were down-regulated). Eight of the 13 SR3-specific DEPs behaved in the same way (six were up-regulated, and two were down-regulated). The abundance of several of the DEPs was affected by the type of stress applied. Two stress-induced JN177 DEPs (spots R7 and R50) were present in at least twice the amount under salinity than under drought stress; three SR3 DEPs (spots R14, R43, and R59) also behaved in this way, and one (spot R9) was more intensely expressed under drought than under salinity (supplemental Table 2S3). Overall, only two cultivar-specific leaf DEPs showed a salinity-specific responsive pattern

TABLE II
Selected DEPs in roots and leaves of JN177 and SR3 seedlings grown under salinity and drought stresses
Other identification parameters such as matched queries and mass tolerance are presented in the supplemental data.

Spot ^a	Label ^b	Accession no. ^c	Annotation	Species	Tmm/Emm ^d	Tp/Ep ^e	Expect ^f PMF	Expect ^f MS/MS ^g	Sequence coverage ^h	Threshold score ⁱ	Score ^j	Matched peptides ^k	Start-end ^l	1C:1D:1S:3C:3D:3S ^m
kDa														
R33	-	T02340	G protein β-subunit-like protein	Common tobacco	35.9/39.5	6.38/6.43	3.3e-02	6.1e-06	24/3	66/44	69/64	K ↓ DVLSVAFS/DNR ↓ Q	108-119	1.8:1.1:1.0:0:0:0
R67	P	g1:1749827	Heterotrimeric G protein subunit	<i>Nicotiana plumbaginifolia</i>	43.5/38.0	6.09/5.79	2.9e-02	2.3e-02	23/—	67/—	70/—	R ↓ AYAEAYGEELLR ↓ A	52-63	1.0:2.2:3.7:1.7:1.9:4.1
R06	+	Q6Z6A7_ORYSA	Putative annexin P35	<i>Oryza sativa</i>	35.5/44.7	6.77/5.59	1.7e-03	2.7e-04	37/3	66/38	82/61	R ↓ AYAEAYGEELLR ↓ A	52-63	1.0:8.9:4.2:1.0:1.2:5.8
R64	P	Q83ZU3_ARATH	Putative serine/threonine kinase	<i>A. thaliana</i>	82.1/63.5	6.27/6.67	5.3e-03	—	26/—	66/—	77/—	K ↓ LFWGGGPNDK ↓ G	83-93	1.0:3.5:3.9:2.4:5.8:5.4
R86	M	KCAB_ORYSJ	Probable voltage-gated potassium channel subunit β	<i>O. sativa</i>	36.4/27.8	6.92/6.85	—	1.9e-01	—/8	—/34	—/49	R ↓ LFWGGGPNDK ↓ G	83-93	1.0:7.8:12.4:4.8:10.3:15.3
R02	M	Q5VKX2_9LILJ	F ₁ -ATPase α subunit	<i>Rhizogonum discolor</i>	44.9/43.1	6.92/6.86	—	1.8e-01	—/6	—/38	—/69	R ↓ EAFPGDVFLHSR ↓ L	261-273	3.4:5.3:5.7:1.0:4.9:3.6
								5.3e+00	—	—	—	R ↓ EAFPGDVFLHSR ↓ L	261-273	—
								4.6e-05	—	—	—	R ↓ TGSVDVNSAGKAMLGR ↓ V + oxidation (Met)	59-74	—
R23	+	PWRZAM	H ⁺ -transporting two-sector ATPase	<i>Triticum aestivum</i>	55.2/54.3	5.70/5.83	2.0e-12	2.9e+02	53/5	78/41	96/129	R ↓ EAFPGDVFLHSR ↓ L	295-307	0.0:0:1.0:1.2:1.3
								1.8e-03	—	—	—	R ↓ MTNFTNFQVDEGR ↓ V	18-32	—
								3.8e-03	—	—	—	R ↓ MTNFTNFQVDEGR ↓ V	18-32	—
								3.9e-05	—	—	—	R ↓ MTNFTNFQVDEGR ↓ V	18-32	—
R70	P	T07110	Vacuolar proton ATPase chain E	Potato	46.5/35.5	5.50/6.97	—	—	38/—	66/—	110/—	—	—	0.0:0:1.5:1.4:1.0
R41	P	Q8GTB9_WHEAT	Glutathione transferase F4	<i>T. aestivum</i>	24.9/31.0	6.50/6.20	2.5e-06	—	42/—	66/—	107/—	—	—	0.0:0:1.0:30.7:26.4
R58	P	g1:1272246	Ethylene receptor	<i>Solanum lycopersicum</i>	78.2/75.5	6.92/6.82	4.9e-06	—	26/—	68/—	71/—	—	—	1.0:4.2:8.3:1.2:1.2:2.1
R72	P	Q8W1S8_MAIZE	DWARF3	<i>Zea mays</i>	44.4/17.4	9.82/5.36	2.8e-02	—	34/—	64/—	64/—	—	—	0.1:0.3:2.0:0:10.4
L01	M	T02253	Chlorophyll a/b-binding apoprotein CP24 precursor	<i>Z. mays</i>	27.0/30.3	9.2/7.81	—	2.9e-06	—/7	—/38	—/80	R ↓ TAENFANFTGEQGYGGK ↓ F	170-187	3.8:2.0:2.2:1.0:1.2:7.6:1
L12	-	PLDA1_MAIZE	Phospholipase D α 1	<i>Z. mays</i>	92.2/87.5	5.38/6.30	3.5e-02	1.10e-02	48/1	66/33	68/40	R ↓ YDQYHSLFR ↓ T	369-378	1.0:2.5:2.5:1.2:3.7:3.4
L14	-	Q95AW6_9LILJ	Rubisco large subunit (fragment)	<i>Cyclanthus bipartitus</i>	43.3/29.4	6.58/6.26	1.7e-02	2.60e-05	30/6	78/44	83/133	R ↓ EMTLGFVOLLR ↓ D	329-339	1.0:4.6:6.8:1.0:3.8:3.6
								1.10e-03	—	—	—	R ↓ EMTLGFVOLLR ↓ D	329-339	—
								3.20e-03	—	—	—	K ↓ TFOGPHGIVQKR ↓ D	136-148	—
								9.80e-01	—	—	—	K ↓ TFOGPHGIVQKR ↓ D	136-148	—
L22	+	Q33289_9LILJ	Rubisco (fragment)	<i>Zingiber gramineum</i>	49.7/25.3	6.79/6.65	4.1e-02	1.70e-01	15/7	66/44	68/172	R ↓ DPQLLLHHR ↓ A	276-285	1.0:14.6:15.2:1.5:12.7:10.6
								2.60e-04	—	—	—	R ↓ EMTLGFVOLLR ↓ D	330-340	—
								1.10e-02	—	—	—	R ↓ EMTLGFVOLLR ↓ D	330-340	—
								4.90e-03	—	—	—	K ↓ TFOGPHGIVQKR ↓ D	137-149	—
								4.70e+00	—	—	—	K ↓ TFOGPHGIVQKR ↓ D	137-149	—
L37	P	Q5IW74_9POAL	Rubisco large subunit (fragment)	<i>Distichlis spicata</i>	44.3/44.8	6.29/6.89	3.1e-11	—	32/—	66/—	159/—	—	—	1.0:18.5:20.5:5.3:12.7:3.6
L42	P	RKWTLG	Rubisco large chain (fragment)	<i>T. aestivum</i>	49.6/30.7	7.07/6.84	9.9e-12	—	30/—	66/—	164/—	R ↓ DPQLLLHHR ↓ A	276-285	0.9:4.30:2.1:0.8:8.9:7
L45	+	Q6U106_9ASPA	Rubisco large subunit (fragment)	<i>Vanilla planifolia</i>	49.1/31.8	6.33/6.55	3.1e-18	3.40e-01	37/8	66/40	229/98	R ↓ FVFCAEAYK ↓ A	208-217	1.0:20.3:21.4:16.8:18.4:12.2
								1.10e-02	—	—	—	R ↓ GGLDFTKDDENVNSQPFMR ↓	185-203	—
								2.20e+01	—	—	—	R ↓ GGLDFTKDDENVNSQPFMR ↓ W	185-203	—
								7.70e+00	—	—	—	R ↓ GGLDFTKDDENVNSQPFMR ↓ W	185-203	—

TABLE II—continued

Spot ^a	Label ^b	Accession no. ^c	Annotation	Species	Tm ^m /Em ^m ^d	Tp ⁱ /Ep ⁱ ^e	Expect ^g PMF ^f	Expect ^g MS/MS ^g	Sequencing coverage ^h	Threshold score ⁱ	Score ^j	Matched peptides ^k	Start-end/ 1C:1D:1S:3C:3D:3S ^m
L46	P	RKWTLC	Ribulose-bisphosphate carboxylase (EC 4.1.1.39) large chain, wheat chloroplast (fragments)	<i>T. aestivum</i>	49.6/30.1	7.07/6.63	3.1e-07		20/—	66/—	119/—		0.5.6:1.1.5:1.0.2.7.7.9
L47	P	Q84QW0_ORYSA	Putative GTP-binding protein (RAB11G)	<i>O. sativa</i>	24.3/26.9	4.96/5.87	3.2e-02		23/—	66/—	69/—		0.1.0.4.6:0.1.8.2.6
L05	P	Q6ZJ14_ORYSA	Calcineurin-like phosphoesterase family-like (fragment)	<i>O. sativa</i>	37.4/30.6	9.12/5.84	3.7e-02		27/—	66/—	68/—		1.0.1.2.8.5.2.4:1.6.3.1
L57	P	T06478	Catalase	<i>T. aestivum</i>	57.0/42	6.52/6.41	5.1e-05		31/—	78/—	108/—		0.0.0.1.0:1.4:1.3

^a The number of the proteins on gel. R, roots; L, leaves.

^b +, the identification of MS/MS was consistent with that of PMF; —, the identification of MS/MS was not consistent with that of PMF, and the former identification was considered to be credible; P, the identification of PMF was credible, whereas that of MS/MS was not; M, the identification of MS/MS was credible, whereas that of PMF was not.

^c The NCBItr, MSDB, or Swiss-Prot reference number of the spot.

^d The molecular mass of the predicted protein/the molecular mass of the protein as estimated by its gel migration. When the identification of MS/MS was consistent with that of PMF, the predicted molecular mass of MS/MS identification is presented.

^e The pI of the predicted protein/the pI of the protein as estimated by its gel migration. When the identification of MS/MS was consistent with that of PMF, the predicted pI of MS/MS identification is presented.

^f p value of identification using PMF data; a value that was less than 0.05 was considered to be credible.

^g p value of identification using MS/MS data.

^h Percentage of predicted protein sequence covered by matched sequences using PMF and MS/MS data. The number before and after the symbol “/” means the coverage obtained from PMF and MS/MS data, respectively. — before and after /, no credible identification using PMF and MS/MS data, respectively.

ⁱ The threshold score of credible identification using PMF and MS/MS data. The number before and after the symbol “/” means the coverage obtained from PMF and MS/MS data, respectively. — before and after /, no credible identification using PMF and MS/MS data, respectively.

^j The statistical probability of a true positive identification of the predicted protein as calculated by MASCOOT using PMF and MS/MS data. The number before and after the symbol “/” means the coverage obtained from PMF and MS/MS data, respectively.

^k ↓, cleavage site.

^l The start and end positions of the identified peptide fragment in the protein sequence using MS/MS data.

^m The ratios between the accumulation of the protein in non-stressed JN177, drought-stressed JN177, salinity-stressed JN177, non-stressed SR3, drought-stressed SR3, and salinity-stressed SR3, respectively. “0” represents no expression.

FIG. 2. A sample of SR3-specific root DEPs. 41, glutathione transferase F4; 59, peroxidase 10; 70, vacuolar proton ATPase subunit E; 71, peroxidase precursor.

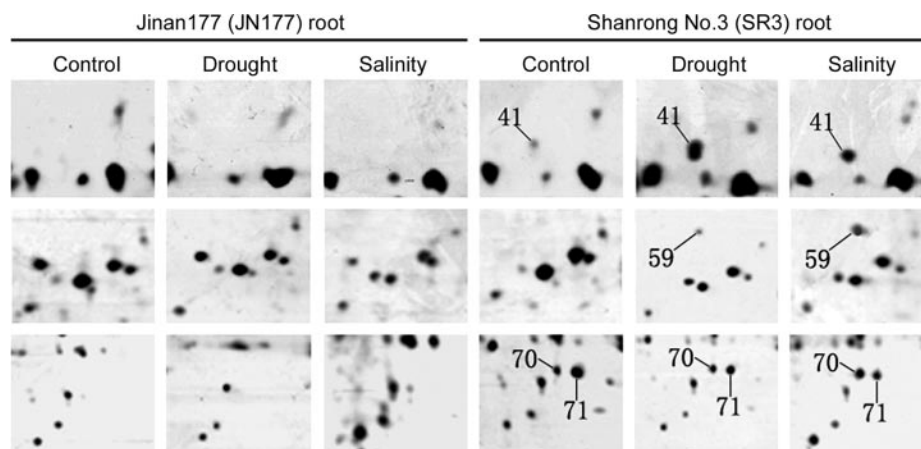


TABLE III

Variety-specific proteins in Shanrong No. 3 and Jinan 177

Up, up-regulated DEPs under salinity and/or drought stress; Un, DEPs showing no response to salinity and/or drought stress in either JN177 or SR3; Down, down-regulated DEPs under salinity and/or drought stress. Overlapped, DEPs showing the same expression patterns in response to both drought and salinity stress in roots/leaves of JN177 or SR3.

Expression patterns	JN177		SR3	
	Leaf	Root	Leaf	Root
Drought				
Up	0	7	0	6
Un	2	7	2	5
Down	0	4	1	2
Salinity				
Up	0	7	1	6
Un	2	7	2	5
Down	0	4	0	2
Overlapped				
Up	0	7	0	6
Un	2	7	2	5
Down	0	4	0	2

(spots L38, which was down-regulated, and L56, which was induced) (supplemental Table 2S6).

Comparison between Proteome and Transcriptome—A cDNA microarray assay was conducted in parallel with the proteomics analysis. Under salinity stress, 3931 sequences showed differential expression in the roots of JN177, and 2093 sequences showed differential expression in the roots of SR3. Of these, 620 (339 up-regulated and 281 down-regulated) were identified in both cultivars. A similar analysis of the JN177 and SR3 leaf transcriptomes produced, respectively, 1557 and 1848 differentially expressed sequences of which 562 (301 up-regulated and 261 down-regulated) were in common. When exposed to drought stress, of the 9099 (JN177) and 4123 (SR3) sequences showing altered expression in the root, 1212 (530 up-regulated and 682 down-regulated) were in common; and in the leaf, of 4402 (JN177) and 6174 (SR3) sequences, 1222 (959 up-regulated and 263 down-regulated) were in common. Of the 88 root and 60 leaf identified DEPs, 74 and 43, respectively, were found to have

matched probes in the microarray (supplemental Table 5S). Of these 74 root DEPs, 25 and 20, respectively, responded similarly at both the mRNA and the protein levels in JN177, whereas 16 (including spot R58, an ethylene receptor) responded to both drought and salinity stresses (Table V). By comparison, in SR3, only 20 (27.0%) and 16 (21.6%) DEPs were found to be correlative between transcriptional and translational levels under drought and salinity stresses, respectively; among these, 13 (17.6%) were co-correlative (Table V). In all, in the absence of stress, 17 DEPs could be identified jointly at both the transcriptional and translational levels, but the equivalent number in the stressed plants was 32. The 45 leaf DEPs followed the same trend (Table V).

A sample of 25 root DEPs was taken to perform RT-PCR to further characterize patterns of gene expression. Distinct patterns of transcription in the root and/or leaf could be demonstrated for 14 of these (Fig. 3). There was a correlation between protein abundance and RT-PCR signal for four genes in drought-stressed JN177 and eight genes in SR3, and for 10 and 11 genes, respectively, under salinity stress (supplemental Tables S2 and S3). Three proteins (spots R69, R85, and R86) (Table II and supplemental Table 2S1) behaved similarly in response to salinity and drought stresses in JN177 and SR3. Overall, the correlation between the transcriptome and proteome was rather poor.

DISCUSSION

Given that drought and salinity are the two most common abiotic stresses affecting crop productivity, improving our understanding of the plant response to these environmental challenges is a major research priority. We have previously used a proteomics approach to expose the response to salinity stress of SR3 and its parent JN177. Here, we extended this analysis to include the response to drought stress, an approach that has uncovered both similarities and differences in how wheat behaves when challenged with these two related but distinct abiotic stresses.

Differences and Similarities in Responses to Drought and Salinity Stress—The functions of the abiotic stress-induced

TABLE IV
Comparison of salt/drought-responsive DEPs in roots and leaves of JN177 and SR3 seedlings

Up, up-regulated DEPs under salinity and/or drought stress; Un, DEPs showing no response to salinity and/or drought stress in either JN177 or SR3; Down, down-regulated DEPs under salinity and/or drought stress. Overlapped, DEPs showing the same expression patterns in response to both drought and salinity stress in roots/leaves of JN177 or SR3 or between roots/leaves of JN177 and SR3 in response to drought or salinity stress.

Stress	JN177			SR3			Overlapped		
	Up	Un	Down	Up	Un	Down	Up	Un	Down
Root									
Drought	33	21	5	32	21	6	21	10	3
Salinity	43	7	9	39	12	8	32	2	7
Overlapped	32	5	1	31	11	3			
Leaf									
Drought	34	20	5	33	21	5	22	12	3
Salinity	38	7	14	29	18	12	21	1	9
Overlapped	33	4	4	25	10	4			

TABLE V

A comparison between proteome and transcriptome of JN177 and SR3 seedlings subjected to no stress and drought or salinity stress

Co-cor, the number of DEPs that showed correlative expression patterns between transcription and translation under both salinity and drought stresses.

Stress	Roots		Leaves	
	Number	Percentage	Number	Percentage
JN177				
Drought	25	33.8	17	40.5
Salinity	20	27.0	13	31.0
Co-cor	16	21.6	9	21.4
SR3				
Drought	20	27.0	13	31.0
Salinity	16	21.6	12	28.6
Co-cor	13	17.6	5	11.9
JN177 vs. SR3				
Control	17	23.0	17	40.5
Drought	32	43.2	22	52.4
Salinity	32	43.2	24	57.1

wheat DEPs were varied, including signal transduction, gene transcription, detoxification, and both carbon and nitrogen metabolism. It is commonly supposed that the primary effect of both drought and salinity is to impose osmotic stress and that plants respond to the imposed disruption in cell homeostasis and ion distribution by the activation of similar signaling pathways and therefore cellular responses to maintain their growth (16–20). Consistent with this model, the majority of both the root and leaf stress-responsive DEPs were shared, whether the imposed stress was drought or salinity (Table II and supplemental Tables 2S3 and 2S6). At both the transcriptional and phenotypic levels also, the two stresses generated a similar set of effects on wheat seedlings (Tables I and V). Similar findings have been reported in other plant species. For example, in *A. thaliana*, 70% of genes strongly induced by salinity were also up-regulated by the imposition of drought stress (21), and a substantial overlap has been noted in induced gene expression in response to drought and salinity stress in rice (16).

The effects of salinity stress, however, are more far reaching than those imposed by drought stress because of the

additional load imposed by ionic stress. Chlorophyll content, transpiration rate, and cellular ionic (Na^+ and K^+) concentration were all modulated more by salinity than by drought stress (Table I). In a comparison of the osmotic (sorbitol-induced) and salinity responses of *A. thaliana* cultured cells, it was concluded that all the proteins responsive to sorbitol stress also showed salinity stress-responsive patterns (6). However, the finding was not reproduced in this study where none of the salinity-induced DEPs were up-regulated by drought stress, and only one of the drought-induced DEPs (spot L35 in SR3) was up-regulated by salinity stress (supplemental Table 2S6). Moreover, there were fewer drought-specific than salinity-specific DEPs (Table IV). Our conclusion is therefore that there are both similarities and differences in the response of wheat to salinity and drought. The differences involve mainly (ionic) stress perception and transduction, whereas the elimination of toxic by-products and metabolic adaptation processes were shared.

Systematic Stress Response Patterns of Leaves and Roots—Although the leaf and the root differ radically with respect to both morphology and biological function, they nonetheless share some metabolic and physiological adaptations to abiotic stress. At the transcriptional level, each produces a distinct transcriptome in response to salinity, drought, and other stresses (22, 23), but some changes at the proteomic level are shared between the two organs. The function of the shared DEPs included signal transduction, transport, detoxification, and both carbon and nitrogen metabolism. Among these were a G protein β -subunit-like protein, a heterotrimeric G protein subunit, a putative annexin P35, a putative serine/threonine kinase (spots R33, R67, R6, and R64; Table II), putative GTP-binding protein, calcineurin-like phosphoesterase family-like, and phospholipase D α 1 (spots L47, L5, L12; Table II) in leaves. As noted previously (11), R33, R67, and L47 are all components of heterotrimeric G protein, which forms part of the Ca^{2+} signal cascade; whereas R6, R64, L5, and L12 all interact with bivalent calcium, and all are capable of binding to phospholipids/phosphoinositides in a Ca^{2+} -dependent manner. The phospho-

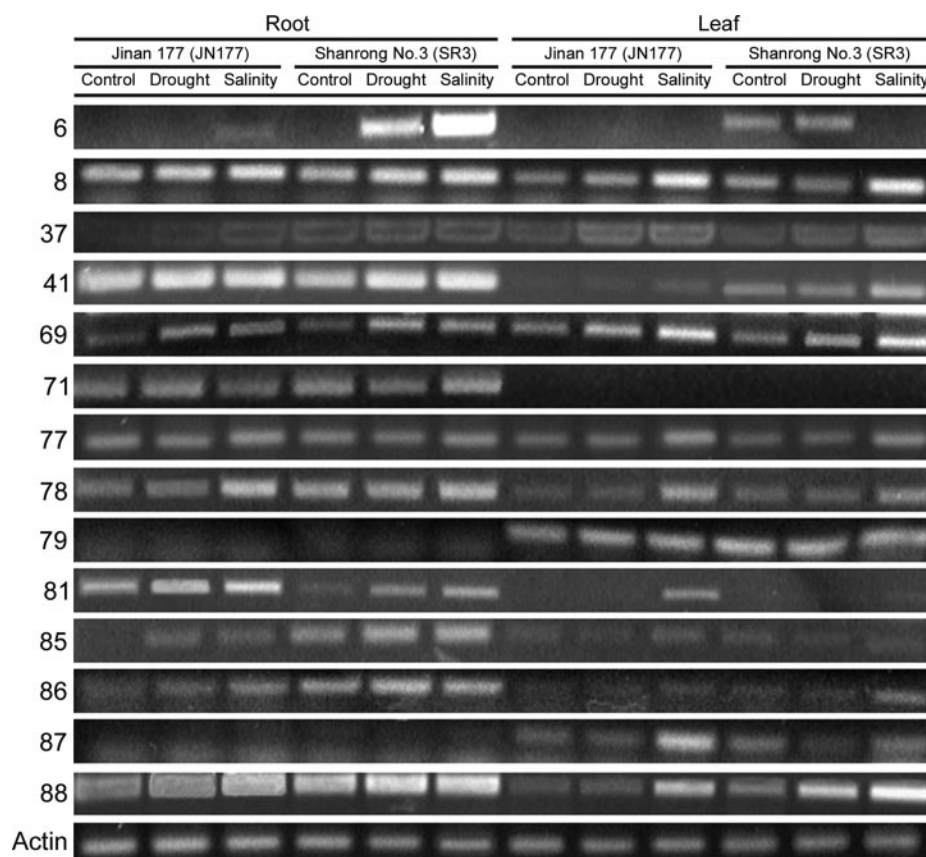


FIG. 3. Gene expression analysis (by RT-PCR assay) of a sample of DEPs. Numbers, the root DEPs; *ACTIN*, the internal standard; *Root*, the transcripts of these DEPs in roots; *Leaf*, the transcripts of these DEPs in leaves.

inositide signal transduction pathway is of major importance in the plant response to salinity and drought stresses (19).

Basis of Enhanced Abiotic Tolerance of SR3—The genetic dissection of salinity and drought tolerance has shown that both are under polygenic control, involving both genetic and genotype-and-environment interactions (24). The ability of a plant to alleviate the buildup of reactive oxygen species (ROS) is an important component of tolerance because ROS disturb cellular homeostasis, thereby causing direct and often irreversible damage to the plant (25–27). ROS scavenging is performed by various antioxidants, which a stressed plant generates as an early response to stress (28–30). Thus, an enhanced ability to generate a greater quantity and/or a greater variety of antioxidants represents a potential means of generating better stress tolerance. Glutathione transferase F4 (spot R41) was expressed in SR3 but not in JN177 and was substantially up-regulated in the presence of either salinity or drought stress (Table II and Fig. 2). Some of the antioxidants present in both cultivars were more abundant in SR3 than in JN177 both in the root and the leaf. The ethylene receptor (spot R58) was, in contrast, more abundant under salinity stress in the roots of JN177 (Table II) as noted previously (11). ROS are known to be capable of activating certain ethylene receptors (31, 32). Thus, the lesser presence of ROS in SR3 may serve to reduce the activation of the ethylene receptor mediating the ROS signal pathway in comparison with JN177.

In support of this notion, the activity under salinity/drought stress of both superoxide dismutase and peroxidase in SR3 was significantly greater than in JN177 (33).

Both ion uptake and water balance were more stable in SR3 than in JN177. Na^+/K^+ transport and vacuolar compartmentalization are required to maintain H^+ transport, which is dependent on ATPases to function for the provision of energy. Cytosolic pH homeostasis, which is also vital for the activity of most enzymes (34), requires the efficient pumping of H^+ across the plasma and the tonoplast membranes (34–37). The survival of plant cells challenged by various abiotic stresses has been shown to strongly depend on V-ATPase activity (38). The expression of both the vacuolar proton ATPase subunit E and the H^+ -transporting two-sector ATPase (spots R70 and R23) was constant in SR3 but not in JN177 roots (Table II, supplemental Table 2S3, and Fig. 2), although cDNA microarray analysis showed that both encoding genes were transcribed at a constant level in the roots of both cultivars (supplemental Table 5S). Thus, the SR3-specific detection of these two proteins suggests the occurrence of either translational regulation and/or some post-translational modification.

SR3 appears better able to assimilate energy than JN177 under salinity or drought stress. Plants typically respond to abiotic stress by shifting from normal development and growth to survival via various alterations in the energy assim-

ilation process (35–37). In particular, photosynthesis is down-graded by Rubisco subunit fragmentation (39–41). The abundance of the fragmented Rubisco subunits was greater in JN177 than in SR3 leaves under drought and salinity stress conditions (Table II and supplemental Table 2S6). Similarly, the photosynthetic rate and the chlorophyll *a* content in SR3 leaves were higher than in the leaves of JN177 (Table I). This higher capacity to maintain photosynthesis under stress may be achieved through a combination of a more robust cellular homeostasis and a more effective means of removing ROS and other toxic by-products. The abundance of the chlorophyll *a/b*-binding apoprotein CP24 precursor (spot L1; Table II and supplemental Table 2S6) in the roots of SR3 was 5-fold that in JN177 leaves under both drought and salinity stresses. This protein is a member of a chlorophyll binding complex that protects chlorophyll in cells and plays an important role in photosynthesis (42). Its higher abundance could contribute to the higher chlorophyll content in the leaves of SR3 seedlings grown under salinity stress (Table I). Furthermore, the higher total soluble sugar (especially sucrose) content of the leaf may have provided SR3 with additional osmoprotectants as well as a larger reserve of carbohydrate to aid recovery when the stress was removed.

The most important manifestation of tolerance is the potential of a plant to recover from stress. The endogenous hormones gibberellin and ethylene are two key regulators of plant growth with positive and negative impacts, respectively (19, 43, 44). One of the SR3 salinity-induced DEPs was DWARF3, a component of the gibberellin biosynthetic pathway. It is possible therefore that the enhanced amount of DWARF3 protein (spot R72; Table II and supplemental Table 2S3) present in the salinity-stressed SR3 seedling contributes to its higher potential to recover after the stress is alleviated. Consistently, an ethylene receptor was more highly up-regulated in JN177 than in SR3, which also implied the higher potential of SR3 for growth recovery through lower sensitivity of the ethylene response. Both fresh and dry biomass and root length of SR3 plants were all superior to those of JN177 plants challenged by stress (Table I). The pattern of DWARF3 gene expression following various periods (0, 1, 3, 6, 12, and 24 h, respectively) of salinity stress showed that transcription decreased within 6 h of the imposition of stress and thereafter recovered gradually (RT-PCR data not shown). Thus, the restriction of normal growth of the wheat seedling caused by exposure to stress appears to be mediated by a rapid reduction in gibberellin synthesis, but in the recovery stage, gibberellin synthesis is restored. Taken together, the proteomics analysis gave us a comprehensive insight into the systematic basis of the high salt and drought tolerance of SR3 that may be a global capacity ranging from intracellular homeostasis reconstruction and ROS and toxicant clearance to energy and carbon assimilation as well as growth recovery.

Using cytogenetic and molecular methodologies, we have found that some genetic materials are introgressed into and

remain stable in the genome of SR3 (45), and we detected novel alleles related to salt and drought stress in SR3 from tall wheatgrass or the recombination/variation of the parent genes (data not shown) based on DEPs or cDNA microarray. Therefore, these data can be used in wheat and in crop improvement through molecular assisted breeding and genetic engineering approaches.

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